H3R42me2a is a histone modification with positive transcriptional effects

Fabio Casadio[a], Xiangdong Lu[b], Samuel B. Pollock[c], Gary LeRoy[d], Benjamin A. Garcia[d], Tom W. Muir[c], Robert G. Roeder[b], and C. David Allis[a,1]

[a]Laboratory of Chromatin Biology and Epigenetics, The Rockefeller University, New York, NY 10065; [b]Laboratory of Biochemistry and Molecular Biology, The Rockefeller University New York, NY 10065; [c]Department of Chemistry, Frick Chemistry Laboratory, Princeton University, Princeton, NJ 08544; [d]Department of Biochemistry and Biophysics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104

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Histone posttranslational modification leads to downstream effects indirectly by allowing or preventing docking of effector molecules, or directly by changing the intrinsic biophysical properties of local chromatin. To date, little has been done to study posttranslational modifications that lie outside of the unstructured tail domains of histones. Core residues, and in particular arginines in H3 and H4, mediate key interactions between the histone octamer and DNA in forming the nucleosomal particle. Using mass spectrometry, we find that one of these core residues, arginine 42 of histone H3 (H3R42), is dimethylated in mammalian cells by the methyltransferases coactivator arginine methyltransferase 1 (CARM1) and protein arginine methyltransferase 6 (PRMT6) in vitro and in vivo, and we demonstrate that methylation of H3R42 stimulates transcription in vitro from chromatinized templates. Thus, H3R42 is a new, "nontail" histone methylation site with positive effects on transcription. We propose that methylation of basic histone residues at the DNA interface may disrupt histone:DNA interactions, with effects on downstream processes, notably transcription.

Eukaryotic genomic DNA is packaged in the form of chromatin, which contains repeating nucleosomal units, consisting of roughly two superhelical turns of DNA wrapped around an octamer of core histone proteins composed of four histone species: one histone H3/H4 tetramer and two histone H2A/H2B dimers (1, 2). Histones are highly basic, globular proteins rich in lysine and arginine residues, with unstructured N-terminal "tail" regions protruding outside the nucleosome structure and structured "core" domains in the DNA-associated portion (3).

Histones are densely decorated with posttranslational modifications (PTMs), such as methylation, acetylation, phosphorylation, ubiquitination, etc. It has been suggested that a given collection of PTMs on one or more histones may contribute to the creation of a "histone code" that modulates gene expression, regulates chromatin structure, and dictates cellular and epigenetic identities during development, therefore extending the information potential of the genetic code encoded in DNA (4, 5). The histone-code hypothesis predicts the existence of "reader proteins" that recognize chromatin covalent-modification marks to influence downstream events through recruitment or stabilization of chromatin-templated machinery ["*trans*" effects (6)], but some PTMs may act directly by affecting the interaction between DNA and histone octamer ["*cis*" effects (7, 8)].

Methylation of lysine residues in histones has been extensively studied, leading to considerable progress in identifying enzyme systems responsible for bringing it about, as well as its function in chromatin-mediated activation and silencing events. Less attention has been paid to methylation of arginine residues in histones, in part, because the low abundance of the modification compared with that on lysines. Although it is increasingly appreciated as a critical epigenetic component in maintaining proper transcriptional regulation during organismal development (9–12), few studies have investigated the biochemical mechanisms by which arginine methylation regulates transcription in a chromatin setting (13–16).

Arginine can be mono- or dimethylated, the latter either in a symmetric (me2s) or asymmetric (me2a) form (17). Enzymes that mediate arginine methylation are protein arginine methyltransferases (PRMTs), with type I and type II PRMTs catalyzing asymmetric and symmetric dimethylation, respectively (17, 18). The human genome encodes nine characterized PRMTs, six of which (PRMT1/8, -2, -3, -4, and -6) are type I PRMTs (17). PRMTs methylate many cellular proteins, and their major targets on histones include histone H4, arginine 3 (H4R3) and histone H3, arginine 2, -17, and -26 (H3 R2, R17, R26) (19).

Although much research has been devoted to PTMs embedded in the flexible N-tails of histones, the role of PTMs situated within the globular domains of the proteins is less well understood. In particular, how PTMs in these domains affect chromatin-templated processes remains unclear. For example, methylation of specific arginine residues in the N-tails of H3 and H4, catalyzed by the coactivators, CARM1 and PRMT1, respectively, is critical in bringing about robust transcription from chromatin templates (15). Whether arginines in the core domains of H3 and H4 exert similar effects on transcription is not clear, even though they play essential roles in the folding of DNA into a nucleosome core particle (20). In particular, arginine residues are the most frequent hydrogen-bond donors to backbone phosphate groups and to thymine, adenine, and guanine bases (21), and the conformation of histones in the nucleosome places several arginines such that their side chains are inserted into the DNA minor groove at every turn of the double helix (3). Intriguingly, addition of a methyl group to an arginine residue not only adds steric bulk but also removes a potential hydrogen bond donor, suggesting a role for this PTM in the regulation of the histone:DNA interaction. Although methylation of arginine residues in the nucleosome core region has been documented (8, 22), to our knowledge, no study to date has investigated the enzyme systems involved in depositing these modifications and their downstream effects.

In this study, we focused our attention on "internal" arginine residues and attempted to discover unique methylation sites. We report the identification of the asymmetric dimethylation on arginine 42 of histone H3 (H3R42me2a) in human and mouse cells, and we identify the methyltransferases for this site, CARM1 and PRMT6. H3R42 is at the DNA entry and exit point of the nucleosome (Fig. 1*A*), a region important for regulating access to the associated DNA (23, 24). Interestingly, Boeke and coworkers recently showed that the residue at position 42 in *Saccharomyces cerevisiae* H3, a lysine in this organism, is methylated in vivo and that mutation of H3K42 to alanine (K42A)

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[1]To whom correspondence should be addressed. E-mail: alliscd@rockefeller.edu.

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Fig. 1. H3R42me2 is a histone mark at the DNA entry/exit region of the nucleosome. (*A*) Crystal structure of the *Xenopus laevis* nucleosome (Protein Data Bank ID code 1KX5) highlighting histone H3 Arg-42 (circled dark blue spheres). (*B*) Sequence alignment of residues 34–52 of histone H3 from multiple eukaryotic species. The residue at position 42 is highlighted. (*C*) MS/MS spectrum of the +2-charged precursor ion at 558.831 *m/z* corresponding to the propionylated/tryptic dimethylated peptide (amino acids 41–49) Pr-YRme2PGTVALR (Arg42-dimethylated) from histone H3. The sequence of the peptide and predicted b and y ions is the *Inset*. Those ions observed in the spectrum are underlined. In the spectrum, the corresponding b ions are labeled in red, and y ions are labeled in blue. Evidence for dimethylation of Arg42 is provided by the b2 ion at 404.3 *m/z* and the y8 ion at 897.7 *m/z*.

results in increased transcriptional output (25). Because most H3 proteins contain arginine at position 42 (Fig. 1*B*), we predicted that methylation of this residue is part of a conserved mechanism used to modulate the interaction between DNA and the histone octamer and, thus, transcription. Using in vitro-transcription assays, we demonstrate that nucleosomes containing H3R42me2a, installed by a three-piece, semisynthetic ligation strategy, can stimulate transcription of the associated DNA. Taken together, these findings suggest that CARM1 and PRMT6 (and potentially other arginine methyltransferases) may stimulate transcription by disruption of the DNA:histone interaction through the methylation of "internal" arginines in core histones. This contrasts with effector-mediated, *trans* activity of many well-documented PTMs in N-tails. Using H3R42 as an example, we envision that methylation at strategic histone core sites such as this serves to modulate histone:DNA interactions, ultimately leading to changes in downstream chromatin structure and function, including in transcription. Our findings are consistent with the notion of "regulated nucleosome mobility" put forward by Cosgrove et al. (8).

Results

H3R42 Is Dimethylated in Vivo. Motivated by the known link between histone arginine methylation and transcription, as well as the understudied nature of this modification relative to lysine methylation, we sought to identify unique arginine methylations on mammalian histones. Given the low abundance of arginine methylation, we imagined that an enrichment step might be a productive route to new examples of the modification on histones. Mann and coworkers, for example, reported a method to identify unknown arginine methylation sites on proteins based on enrichment with antibodies targeted to methylated residues followed by liquid chromatography–tandem mass spectrometry (MS/MS) analysis (26). We adopted the same strategy and immunoprecipitated chromatin-associated, acid-extracted histones from HeLa cells with commercially available antibodies recognizing proteins monomethylated and dimethylated on arginine residues (Abcam ab412). Using electrospray MS/MS, we identified the dimethylated form of H3R42 in the immunoprecipitated material. Fig. 1*C* shows the MS/MS spectrum demonstrating the dimethylated tryptic peptide containing H3R42me2.

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CARM1 and PRMT6 Methylate H3R42. To identify the methyltransferase(s) responsible for methylating H3R42, we overexpressed and immunoprecipitated all of the known human arginine methyltransferase enzymes, PRMT1-2-3-5-6-7-8 and CARM1(PRMT4), in HEK293 cells. Each was then assayed for methyltransferase activity against a histone H3 peptide centered on R42 (residues 34–52) using tritiated S-adenosyl methionine (SAM) as a radioactive methyl donor. Under these assay conditions, only CARM1 and PRMT6 were able to methylate the H3 peptide substrate (Fig. 2*A*). In agreement with previous reports (17), all of the enzymes except PRMT2 showed activity in a control reaction using recombinant human histones as substrates (Fig. S1). Because both CARM1 and PRMT6 are only capable of dimethylating asymmetrically (17), we conclude that the dimethylation of R42 observed by MS is likely asymmetric (H3R42me2a).

CARM1 and PRMT6 are known to methylate histones, and their previously identified target sites are all located within the H3 N-tail: H3R17, H3R26, and H3R2, respectively (17). PRMT6 has been shown to add a methyl group to a monomethylated target more efficiently than to an unmodified one (27, 28). We, therefore, wished to both confirm methylation by CARM1 and PRMT6 on H3R42, and assay substrate specificity, using recombinant enzymes and H3 peptides (residues 34–52) harboring unmodified, mono- or dimethylated R42 as substrates. As a point of reference, we used unmodified H3(1-20) peptides that carry one target site for both enzymes (R17 for CARM1 and R2 for PRMT6). Fig. 2*B* shows the incorporation of radioactive methyl groups onto the different peptides: both enzymes methylated the R42me1 peptide better than the corresponding unmodified peptide, whereas neither could methylate the R42me2a peptide, indicating specificity for the R42 site. We noticed that whereas CARM1 showed comparable activity toward the unmodified H3(1-20) and H3(34-52) peptides, PRMT6 showed higher activity toward H3(1-20), suggesting that R42 might be a less preferred, secondary site for PRMT6.

To test whether CARM1 and PRMT6 are required for H3R42 methylation in vivo, we performed siRNA knockdown of each enzyme alone or both together in HEK293 cells (Fig. 2*C*) and determined the levels of R42 methylation by MS, relative to cells treated with control ("scrambled") siRNA. As shown in Fig. 2*D*, knockdown of either CARM1 or PRMT6 alone resulted in a significant reduction of R42 methylation, and the double knockdown resulted in almost undetectable levels of the PTM. In addition, overexpression of either CARM1 or PRMT6 resulted in elevated levels of R42 dimethylation in HEK293 cells (Fig. S2). We also took advantage of the existence of CARM1-deficient ($CARM1^{-/-}$) mouse embryonic fibroblasts (MEFs) (10) and measured by MS the level of R42 methylation in $CARM1^{+/+}$ or $CARM1^{-/-}$ MEFs. We observed a marked decrease in R42 methylation in the absence of CARM1 (Fig. S3). Taken together, these results demonstrate that CARM1 and PRMT6 methylate H3R42 in vitro and in vivo.

H3R42me2a Directly Stimulates (p53-Dependent) Transcription from a Chromatinized Template. Our data support a role for the transcriptional coactivator CARM1, together with PRMT6, in regulating the level of H3R42 methylation, a site that differs structurally from the other previously characterized methylation sites in the H3 N-tail (R2, R17, and R26). Mutational studies have suggested that these sites have direct stimulatory effects on in vitro transcription from chromatinized templates (15). Because of the critical position of H3R42 within the nucleosome, we speculated that its methylation may disrupt key protein–DNA contacts, allowing the transcription machinery easier access to the DNA and facilitating transcription. With this in mind, we sought to test whether H3R42me2a has a stimulatory effect on transcription.

To this end, seeking to avoid complications of enzymatic addition of H3R42me2a (i.e., incomplete methylation, differences between CARM1 and PRMT6, etc.), we generated a semisynthetic histone H3 protein harboring premodified R42me2a using expressed protein ligation (29). The location of the modified residue, within the middle of the polypeptide sequence, dictated the use of a three-piece protein ligation strategy. As shown in Fig. 3*A*, we assembled the target protein from two synthetic fragments (residues 1–28 and 29–46; peptides **1** and **2**) and one recombinant fragment (residues 47–135; peptide **3**). A key feature of this modular strategy was the incorporation of a cryptic α-thioester, in the form of a hydrazide (30), within the central synthetic peptide harboring the dimethylarginine residue. This facilitated the regioselective assembly of the target protein using an N-to-C sequential ligation procedure in which the hydrazide was converted into an thioester following the first ligation

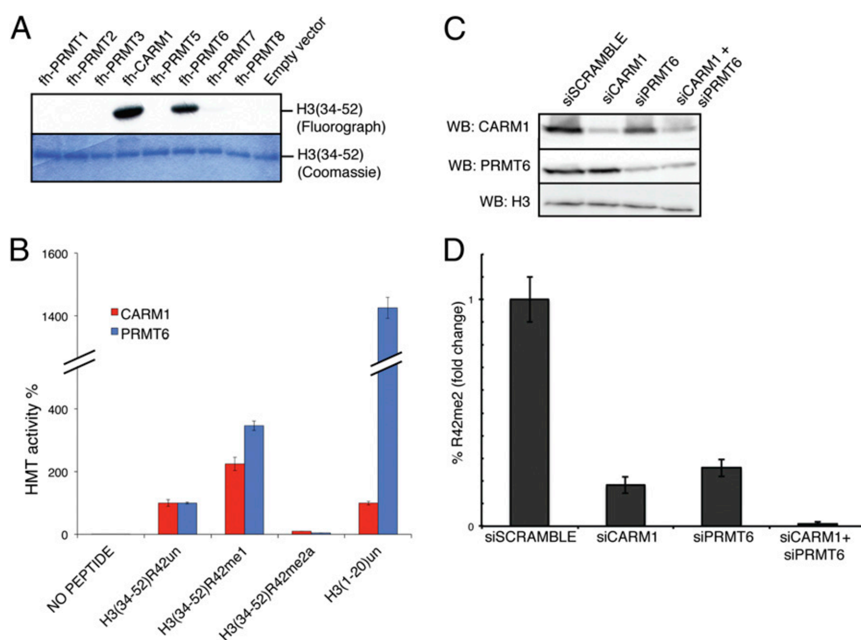

Fig. 2. CARM1 and PRMT6 methylate H3R42 in vitro and in vivo. (*A*) H3 peptide (amino acids 34–52) was incubated with immunoprecipitated Flag-HA-tagged human PRMT1, -2, -3, -5, -6, -7, -8, or -9 or CARM1(PRMT4) in the presence of [^{3}H]methyl-SAM and resolved by SDS/PAGE. Radioactive methyl incorporation is identified by fluorography. (*B*) Comparison of the methyltransferase activity of CARM1 and PRMT6 against different peptide substrates. Activity was measured by scintillation counting in triplicate experiments and is expressed as percentage of the activity measured for H3(34-52) peptides. Baseline (0%) was set as the activity measured in no substrate controls (no peptide). (*C*) Levels of CARM1 and PRMT6 measured by Western blot in HEK293 extracts after treatment with either control siRNA or siRNAs against CARM1, PRMT6, or both. H3 levels are shown as loading control. (*D*) Levels of H3R42 methylation were measured by MS in acid extracts from the same cells as in *C*. The percentage of H3R42 methylation for each sample is expressed in fold changes relative to the siSCRAMBLE control sample.

Fig. 3. Generation of semisynthetic H3R42me2a protein, **6**. (*A*) Schematic representation of the semisynthetic route used. See *Materials and Methods* for details. (*Ai*) N^{α}-Boc solid-phase peptide synthesis; SR is mercaptopropionyl-arginine. (*Aii*) N^{α}-Fmoc solid-phase peptide synthesis. (*Aiii*) Ligation of peptide-thioester **1** to peptide-hydrazide **2** to give intermediate polypeptide **4**. (*Aiv*) Ligation of the thioester derivative of **4** to recombinant protein **3**, followed by radical-mediated desulfurization to give final product **6**. (*B*) ESI-MS spectrum of purified H3(R42me2a), **6**. Charge states are labeled. $(M+H)^+$ observed, 15,253 ± 4 Da; expected, 15,253 Da.

reaction, thereby eliminating the risk of self-reaction of the central fragment and allowing directed ligation of the recombinant fragment. The final step in the synthesis involved conversion of the two cysteine residues at the ligation junctions into the native alanine residues through desulfurization, affording the desired semisynthetic product **6** in good yield and high purity (Fig. 3*B*).

Semisynthetic H3R42me2a was successfully incorporated into core histone octamers with wild-type recombinant H2A, H3, and H4 (Fig. S4*A*). Control histone octamers were formed containing recombinant unmodified H3. These octamers were used to reconstitute chromatin templates on a plasmid DNA (Fig. S4*B*) using a recombinant ACF (ATP-utilizing chromatin assembly and remodeling factor)/NAP1 (nucleosome assembly protein 1) system (31) as described previously (32). Characterization of assembled chromatin templates by micrococcal nuclease digestion (Fig. S4*C*) and DNA-supercoiling assay (Fig. S4*D*) confirmed that methylation of H3R42 does not affect chromatin template properties.

Our previous work demonstrates cooperation between CARM1 and protein (p)300 in p53-dependent gene activation (15). In this study, we tested whether H3R42me2a acts together with p300 to stimulate transcription directly, using the premethylated template in an activator-dependent in vitro transcription system (Fig. 4*A*). In the presence of both p53 and p300, we observed a 2.5-fold increase in transcription from R42me2a over unmodified templates (Fig. 4*B*, compare lane 6 with lane 5). We also performed the reaction in absence of p300; under these conditions, R42 methylation resulted in a ∼sixfold increase in transcription versus the unmodified template (Fig. 4*B*, compare lane 4 with lane 3). Taken together, these results demonstrate a robust and direct transcriptional stimulatory effect by H3R42me2a. We provide evidence that methylation of "internal" arginine residues at the histone:DNA interface can directly stimulate transcription.

Discussion

Methylation of arginine residues on histone tails has been linked to both transcriptional activation and repression (17). Here, we identify an arginine methylation modification on the core domain of H3 (H3R42) and show that a methylation event in the core domain of H3 has a direct transcriptional activating role. By presumably bringing about an intrinsic change to the chromatin structure, the presence of this modification allows for a more efficient transcriptional initiation in our in vitro-transcription assays.

Fig. 4. H3R42me2a stimulates transcription in vitro. (*A*) Experimental scheme of chromatin assembly and transcription. The chromatin templates were assembled with wild-type and premodified H3R42me2 human octamers using NAP1 and ACF/ISWI assembly system. (*B*) p53-dependent, p300-mediated transcription activation in the presence or absence of H3R42 asymmetric dimethylation.

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Methylation of Residue 42 in H3 Is Conserved Through Evolution. Histones are among the most conserved proteins in eukaryotes (33). Residue 42 in histone H3 shows an amino acid change with functional conservation: *S. cerevisiae* "evolved" a lysine at this position, whereas most other organisms have arginine (Fig. 1*B*). Both residues have the potential to create electrostatic interactions or hydrogen bonds with DNA, suggesting that this contact is structurally important. We show here that H3R42 is dimethylated in both murine and human cells and that the methylation is mediated by methyltransferases CARM1 and PRMT6. A recent report found that H3K42 is methylated and plays a functionally important role in transcription in budding yeast, although the responsible methyltransferase was not identified and H3R42 was not detected in mammalian cells in this study (25). Together with our data, these findings suggest that methylation of residue 42 in histone H3, on lysine or on arginine residues, is an important regulatory modification that may regulate the interaction between DNA and histone octamer at a critical site of interaction (3, 23, 24).

H3R42me2a As a Direct Modulator of Transcription. Using a semisynthetic strategy to generate a homogeneous source of H3R42me2a in combination with in vitro-transcription assays, we show that H3R42me2a intrinsically makes chromatin templates better substrates for transcription. We speculate that this increase in transcription is due to the weakening of the interaction between the histone octamer and DNA as a consequence of methylation of H3R42. Several previously reported observations support our hypothesis: (*i*) the DNA entry/exit region is important in controlling the unwrapping of DNA from nucleosomes (34); (*ii*) mutation of residue 42 to alanine makes nucleosomes more mobile (24); and (*iii*) mutation of residue 42 to alanine in *S. cerevisiae* results in an hypertranscription phenotype (25). Future studies could address whether methylation of H3 at R42 directly destabilizes and structurally affects nucleosomes, as has been shown for a small number of other modifications within histone core domains. For example, acetylation of H4K56 increases DNA unwrapping (35), acetylation of H3K122 enhances the rate of nucleosome disassembly upon mechanical stress (36), and ubiquitylation of H2B prevents chromatin compaction (37). In addition, H3R42 methylation might act in an effector-dependent (*trans*) fashion, although to our knowledge, no such "readers" have been identified.

Both CARM1 and PRMT6 Methylate R42 in Vivo. We show here that H3R42 dimethylation has a positive effect on in vitro transcription. The methyltransferase CARM1 was originally identified as a transcriptional coactivator for nuclear steroid receptors (9), and its positive role in transcriptional activation has been well established (15). Conversely, PRMT6 activity has generally been associated with transcriptional repression (27, 38, 39), although it has more recently been reported that this methyltransferase might also play a positive role in regulating the transcription of certain target genes (40).

Our data demonstrate that both these enzymes are important in controlling the overall level of R42 methylation in vivo. Moreover, the knockdown experiments in Fig. 2*D* suggest that CARM1 and PRMT6 are only partially redundant and cooperate, to some extent, to regulate H3R42 methylation. CARM1, in fact, accounts for about 80% of the methylation in vivo, whereas PRMT6 accounts for about 75%, leading us to speculate that the two enzymes might interact or that the activity of one might influence the activity of the other. Further investigations could address this potential interaction. At the same time, given the observed partial overlap, the question remains whether both enzymes are active in the same or different tissues or genomic locations. The order of events of arginine methylation also remains unclear: do CARM1 and PRMT6 deposit methyl groups on their tail sites and on R42 at the same time, in the same genomic location, and in the same cells? It is possible that interaction with different factors might impart different specificities to the enzymes or causing their recruitment to different regions. With respect to this, it is also unclear how these enzymes gain access to the H3R42 site in vivo, given that it is intimately engaged in DNA contacts within the nucleosome (3). Conceivably, the enzymes may be able to trap the intrinsic "breathing" motions within the particle (41). Alternatively, the enzymes may act in conjunction with remodeling or chaperone activities that would transiently expose the substrate sequence. Biochemical experiments will be needed to test these intriguing possibilities.

In conclusion, methylation of H3 at R42 (K42 in budding yeast) has emerged as a PTM in mammalian cells, in keeping with a conserved function in evolution and with a role in stimulating transcription. We demonstrate that R42 can be dimethylated and that CARM1 and PRMT6 are the relevant methyltransferases. We speculate that this mark facilitates a structural alteration in the chromatin, making it easier to transcribe in transcription assays. It is further proposed that H3R42me2a might be a direct modulator of the transcription of target genes at the specific genomic locations where it is deposited. We envision that our observations will be extended to other arginine residues at the interface with DNA within the nucleosome, which might be methylated as well and present similar effects.

Materials and Methods

Quantification of H3R42me2. Quantification of the propionylated/triptic histone H3 peptides (amino acids 41–49) Pr-YRPGTVALR (with or without R42me2) was accomplished by measuring the area under the extracted-ion chromatogram (XIC) peaks corresponding to the +2-charged precursor ions to Pr-YRPGTVALR (544.815 *m/z*) and Pr-YRme2PGTVALR (558.831 *m/z*). Relative quantification was accomplished by comparing such XIC peak data from the Pr-YRme2PGTVALR ion to the Pr-YRPGTVALR ion.

Peptide Synthesis. Amino acid derivatives, chlorotrityl resin, and coupling reagents were purchased from Novabiochem.

Peptide 1. The sequence corresponding to residues 1–28 of human H3.2 was synthesized on a mercaptopropionyl-Arg-phenylacetamidomethyl (PAM) resin, which affords a peptide α-thioester upon cleavage. Chain assembly used manual solid-phase peptide synthesis with a *t*-butyloxycarbonyl (Boc) N^{α} protection strategy and using 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) for amino acid activation. The peptide was cleaved from the resin on a 500 mg of peptide-resin scale by stirring in 10 mL of anhydrous hydrofluoric acid (Sigma) at 0 °C for 1 h with p-Cresol (Sigma) as a scavenger before workup in cold ether, dissolution in 50% (vol/vol) solvent B (45%/55% MeCN/H2O with 0.1% TFA vol/vol), and lyophilization. Cleaved product was purified by reverse-phase (RP)-HPLC, yielding ~35mg of peptide **1**, which was characterized by electrospray MS (measured mass, 3,239.8 Da; predicted mass, 3,239.8 Da).

Peptide 2. The sequence corresponding to residues 29–46 of human H3 containing a Ala29-Cys mutation and was synthesized on 2-chlorotritylhydrazine resin (~0.4 mmol/g), which was derived as described previously (42). The peptide itself was synthesized using manual solid-phase peptide synthesis with a fluorenylmethyloxycarbonyl (Fmoc) N^{α} protection strategy and using HBTU for amino acid activation (Sigma). The peptide was cleaved from the resin on a 500 mg of peptide-resin scale by shaking in 10mL 95:2.5:2.5 TFA:H_2O:triisopropylsilane at room temperature for 1.5 h before rotary evaporation of the TFA and workup in cold ether, dissolution in 50% solvent B, and lyophilization. Cleaved product was purified by RP-HPLC, yielding ~30 mg of peptide **2**, which was characterized by electrospray MS (measured mass, 1,966.1 Da; predicted mass, 1,966.1 Da).

Preparation of H3(47-135)A47C,C110A. A template pET3 plasmid containing hH3.2 was amplified by PCR to afford a fragment containing residues 47–135 with a Ala47-to-Cys mutation. This was inserted into a pET30a(−) vector plasmid containing N-terminal poly-His and SUMO elements and verified by DNA sequencing. *Escherichia coli* BL21(DE3) cells (Invitrogen) transformed with the above His-SUMO-H3(47-135, A47C) construct were grown in Luria–Bertani medium at 37 °C until midlog phase, and protein expression was induced by the addition of 0.6 mM isopropyl thiogalactoside (IPTG) and allowed to continue at 37 °C for 4 h. After harvesting the cells by centrifugation

at 3,000 × *g* for 20 min, the cell pellet was resuspended in lysis buffer (50 mM Tris, 200 mM NaCl, 1 mM EDTA; pH 7.5) and frozen at −80 °C. Thawed cells were lysed by sonication and passage through a French press, and the soluble material was removed by centrifugation at 30,000 × *g* for 30 min. The inclusion bodies, containing the desired fusion protein, were redissolved in lysis buffer plus 6 M guanidinium chloride (GuHCl) for 3 h, the solution was centrifuged as before, and the supernatant was incubated for 1.5 h at 4 °C with Ni-NTA resin (Qiagen) preequilibrated in lysis buffer plus GuHCl. The resin was washed with 10 column volumes (CV) of D500 (6 M GuHCl, 50 mM Tris, 500 mM NaCl, 3 mM β-mercaptoethanol, pH 8.0) and 10 CV D1000 (D500 with 1 M NaCl), followed by 5 CV urea buffer (6 M urea, 50 mM Tris, 150 mM NaCl; pH 7.9). Elution was carried out with 7× 1 mL of elution buffer (urea buffer plus 500 mM imidazole). Fractions were analyzed by SDS/PAGE and combined before immediately undertaking cleavage of the His-SUMO tag. Proteolytic cleavage of the His-SUMO-H3 construct proceeded under the following conditions: the construct was diluted in cleavage buffer to afford a final concentration of 2 M urea, 166 mM imidazole (2 mM DTT, 150 mM L-arginine, 10 mM L-cysteine, 50 mM Tris, 150 mM NaCl; pH 8) and 1 U of Ulp1 SUMO protease per 100 μg of cleavable material. The final concentration of fusion protein was ∼0.25 mg/mL. The solution was left overnight with stirring. The imidazole remaining in solution was removed using 3,000 molecular-weight-cutoff spin concentrators; the uncleaved material was removed by successive depletion on Ni beads. The resulting eluent was purified using preparative HPLC. The identity of the purified protein **3** was confirmed by MS (measured mass, 10,403.3 Da; predicted mass, 10,403.1 Da). This protocol afforded ∼0.5 mg of purified protein per liter of initial bacterial culture.

Ligation and Desulphurization. Ligation of thioester peptides **1** (1 mM) and **2** (2.5 mM) to form polypeptide **4** was carried out in ligation buffer [6 M GuHCl, 0.2 M phosphate (pH 7.0), 50 mM 4-mercaptophenylacetic acid (MPAA), 20 mM *tris*(2-carboxyethyl)phosphine (TCEP)] under argon at room temperature. The reaction was complete after 3 h, and the product was purified by semipreparative HPLC and characterized by electrospray ionization–MS (ESI-MS) (measured mass, 49,44.9 Da; predicted mass, 4,944.8 Da). The hydrazine moiety in **4** was converted into a thioester as described (40). Briefly, polypeptide **4** was added at 2.5 mM to sparged 6 M GuHCl and 0.2 M phosphate at pH 3.0 and cooled to −10 °C before adding 10 mM NaNO2, allowing it to sit for 25 min, and performing thioesterification by the addition of 150 mM MPAA, 1 mM polypeptide 3, and base up to pH 7.0. Following the addition of 30 mM TCEP after 1 h and a complete reaction time of 6 h, full-length histone protein **5** was purified by semipreparative HPLC and characterized by ESI-MS (measured mass, 15,316.7 Da; predicted mass, 15,316.9 Da). The final product, **6**, in which the cysteines at the ligation junctions were converted back to the native alanines, was obtained through radical desulfurization according to established protocols (37). Briefly, protein **5** (0.3 mM) was dissolved in desulfurization buffer (6 M GuHCl, 0.2 M phosphate, 250 mM TCEP; pH 7.0), and the reaction was initiated through the addition of reduced glutathione (final concentration, 30 mM) and VA-061 (final concentration, 16 mM). The desulfurization solution was flushed with argon, wrapped in parafilm, and placed at 37 °C overnight. Semisynthetic protein **6** was then purified out of the solution by semipreparative HPLC purification. Fractions were analyzed by ESI-MS and lyophilized, yielding ∼1 mg of final product (measured mass, 15,252.7 Da; predicted mass, 15,252.8 Da).

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